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Confirmation No. 2649

Applicant: Moschel et al.

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Commissioner for Patents
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DECLARATION UNDER 37 C.F.R. 1.132 FROM MELINDA HOLLINGSHEAD, DVM, PH.D.

Sir:

I, Melinda Hollingshead, hereby declare that:

1. I am an employee of the National Institutes of Health and serve as the Chief of the Biological Testing Branch of the National Cancer Institute at Frederick, MD.

2. I received a Bachelor of Science degree from the University of Alabama in Huntsville with a major in biology and a minor in chemistry. Following graduation, I trained as a Medical Technologist at the Huntsville Cooperative School of Medical Technology and received national certification as an MT(ASCP). After completing a year of post-graduate studies in animal science and nutrition, I matriculated at the Auburn University College of Veterinary Medicine and received a doctorate in veterinary medicine (D.V.M.). After one year in private veterinary practice, I entered post-graduate training at the North Carolina State University School of Veterinary Medicine from which I earned a Ph.D. in Veterinary Medical Sciences with a concentration in Immunology. I spent 5.5 years managing a BSL-3 containment facility at Southern Research Institute conducting animal efficacy studies of new antiviral agents. I joined the Biological Testing Branch of the National Cancer Institute in 1992 and have been continuously employed since that time

working on efficacy evaluations of potential anticancer agents. I have over 100 peer-reviewed publications all of which are in the field of preclinical drug evaluations.

3. Claims 16, 17, 31, 32, 40, 41, and 59-64 of the subject patent application have been rejected under 35 USC 112, first paragraph, for an alleged lack of enablement.

4. Based on studies carried out under my direction or supervision, a compound of the claimed invention, O^4-benzylfolate, decreased tumor growth in mice when co-administered with an antineoplastic alkylating agent, as discussed below. O^4-benzylfolic acid is designated herein as O4BFA or NSC 730793 and the sodium salt of O^4-benzylfolic acid is designated as O4BF or NSC 742482. See Figures 1-2 of this Declaration.

5. Three studies were carried out. In the first two studies, antitumor activity was determined against SW-620 human colon tumors and IGR-OV-1 human ovarian tumors in subcutaneous xenografts alone and in combination with BCNU (NSC 409962). In both studies, mice were placed onto folate deficient diets at least one week prior to initiating therapy as normal mouse folate levels are multi-fold higher than human levels. Placing the mice on folate-deficient diets reduces the plasma folate levels to levels more consistent with humans (confirmed by serum folate quantitation). The SW-620 tumors were grown in female athymic nude (nu/nu NCr) mice while the IGR-OV-1 tumors were grown in female SCID (scid/scid NCr) mice. The experimental design for both studies included treatment with BCNU at 10, 6.7 and 4.5 mg/kg/dose given daily for 5 days (QDx5) by the intraperitoneal (IP) route; O4BFA as a 40 mg/ml solution delivered by subcutaneous (SC) osmotic pump at a rate of 1 μL/hr for 7 days (a daily dose of approximately 48 mg/kg); and combinations of BCNU (10 and 6.7 mg/kg) and O4BFA (40 mg/ml). For these studies BCNU was solubilized in 2% ethanol in saline and the control group was treated with the vehicle alone. There were 20 mice in the control group with 10 mice in each treatment group. Tumors were monitored with bidirectional caliper measurements and tumor weights were calculated as follows:

$$(\text{tumor length}) \times (\text{tumor width})^2 \times 0.5$$

Body weights were measured to assess toxicity. Standard endpoints were calculated from the tumor weights. The results obtained in the SW-620 tumor model are shown in Table 1

and Figure 3. The results obtained in the IGR-OV-1 tumor model are shown in Table 2 and Figure 4.

Table 1: Effect of O4BFA and BCNU on SW-620 Human Colon Tumor Xenografts in Athymic (nu/nu NCr) Mice

Test Conditions	# of Mice	Drug Deaths	max % rel mean net wt Loss (day)	opt %T/C (day)	median days to 300 mg	growth delay %T-C/C	Net Log Cell Kill
2% ethanol in saline QDx5 IP	20	0	no wt loss		13.8		
10 mg/kg NSC 409962 QDx5 IP	10	0	1.8(24)	90(21)	13.9	1	-0.2
6.7 mg/kg NSC 409962 QDx5 IP	10	0	no wt loss	87(21)	13.2	-4	-0.3
4.5 mg/kg NSC 409962 QDx5 IP	10	0	no wt loss	85(21)	13.2	-4	-0.3
40 mg NSC 730793/ml at 1 µL/hr for 7 days SC	10	0	no wt loss	66(21)	14.5	5	-0.4
10 mg/kg NSC 409962 QDx5 IP + 40 mg NSC 730793/ml at 1 µL/hr for 7 days SC	10	0	0.4(21)	85(21)	13.3	-4	-0.4
6.7 mg/kg NSC 409962 QDx5 IP + 40 mg NSC 730793/ml at 1 µL/hr for 7 days SC	10	0	no wt loss	108(21)	12.2	-12	-0.5

Table 2: Effect of O4BFA and BCNU on IGR-OV-1 Human Ovarian Tumor Xenografts in SCID Mice

Test Conditions	# of Mice	Drug Deaths	max % rel mean net wt Loss (day)	opt %T/C (day)	median days to 300 mg	growth delay %T-C/C	Net Log Cell Kill
2% ethanol in saline QDx5 IP	20	0	no wt loss		20		
10 mg/kg NSC 409962 QDx5 IP	10	2	17.7(19)	99(19)	20.7	4	-0.1
6.7 mg/kg NSC 409962 QDx5 IP	10	0	no wt loss	88(19)	23.7	19	0
4.5 mg/kg NSC 409962 QDx5 IP	10	0	13.4(33)	82(19)	21.1	6	-0.1
40 mg NSC 730793/ml at 1 µL/hr for 7 days SC	10	0	no wt loss	81(19)	20.6	3	-0.3
10 mg/kg NSC 409962 QDx5 IP + 40 mg NSC 730793/ml at 1 µL/hr for 7 days SC	10	0	12.5(19)	101(19)	19.5	-3	-0.3
6.7 mg/kg NSC 409962 QDx5 IP + 40 mg NSC 730793/ml at 1 µL/hr for 7 days SC	10	0	2.0(26)	80(19)	22.6	13	-0.2

6. In the third study, the sodium salt of O4BFA, NSC 742482 (O4BF), was tested against IGR-OV-1 human ovarian tumor xenografts in female SCID mice fed a folate-deficient diet for 2 weeks prior to the start of treatment. The experimental design for this study included treatment with BCNU at 10, 6.7 and 4.5 mg/kg/dose given daily for 4 days (QDx4) by the IP route; O4BF at 200 mg/kg/dose given twice starting the day prior to BCNU treatment (Q10Hx2) with the 2 doses spaced approximately 10 hr apart; and combinations of BCNU (10, 6.7 and 4.5 mg/kg) and O4BF (200 mg/kg). BCNU was solubilized in 2% ethanol in saline, the O4BF was solubilized in 0.9% saline, and the control group was treated with 2% ethanol in saline. The results obtained with NSC 742482 in the IGR-OV-1 tumor model are shown in Table 3 and Figure 5.

Table 3: Effect of O4BF and BCNU on IGR-OV-1 Human Ovarian Tumor Xenografts in SCID Mice

Test Conditions	# of Mice	Drug Deaths	max % rel mean net wt Loss (day)	opt %T/C (day)	median days to 300 mg	growth delay %T-C/C	Net Log Cell Kill
2% ethanol in saline QDx4 IP	19	0	0.8(23)		13.7		
10 mg/kg NSC 409962 QDx4 IP	8	0	16.8(23)	72(23)	16.3	19	0
6.7 mg/kg NSC 409962 QDx4 IP	8	0	9.2(23)	68(23)	17.6	28	0
4.5 mg/kg NSC 409962 QDx4 IP	8	0	6.3(23)	69(16)	18.6	34	0.1
200 mg/kg D-S742482 Q10Hx2	11	0	0.6(23)	77(16)	17.4	27	0.1
10 mg/kg NSC 409962 QDx4 IP + 200 mg/kg D-S742482 Q10Hx2	8	0	15.2(23)	70(16)	17.7	29	0
6.7 mg/kg NSC 409962 Qx4 IP + 200 mg/kg D-S742482 Q10Hx2	8	0	5.8(26)	59(23)	21.3	55	0.2
4.5 mg/kg NSC 409962 QDx4 IP + 200 mg/kg D-S742482 Q10Hx2	8	0	1.9(20)	77(16)	17.1	25	0

7. Traditional activity criteria hold that an optimal % T/C (Tumor/Control) value of 40% or less is indicative of antitumor activity. While none of the 3 studies contained any single or combination treatments with %T/C in this range, it is worth noting that there are several suggestions of activity. For example, in the case of SW-620 colon tumor xenografts, single agent therapy with continuous infusion O4BFA resulted in an optimal % T/C of 66% (Table 1) and a demonstrable shift in the slope of the tumor growth curve (see Figure 3, line 1 connecting circles for NSC 730793, 40.0 mg/ml SC, 7 day infusion, Day 8) vs. control line 2 (QD x 5, Day 8, 2% EtOH in saline)). It is also apparent from the weight loss data in Table 1 that continuous exposure to O4BFA at this level (~48 mg/kg/day) is not toxic under the conditions evaluated. Therefore, higher dose levels may result in greater tumor growth inhibition.

8. The study conducted against IGR-OV-1 tumors using bolus dosing with O4BF in combination with BCNU offers evidence of activity in that the combination of 6.7

mg/kg BCNU and 200 mg/kg O4BF group produced a tumor growth delay of 55% (Table 3). This can also be seen graphically in Figure 5 by comparing the control line 2 (QD x 4 days, Day 8, 2% EtOH in saline) to the combination treatment line 1(NSC 742482, 200.0 mg/kg /dose IP, Q10H x 2h, Day 8; NSC 409962, 6.7 mg/kg/dose IP, QD x 4 days, Day 9). This tumor growth delay is greater than the growth delay observed when 6.7 mg/kg BCNU alone was administered (28%, Table 3, column 7). The lack of a significant tumor growth delay at the higher dose of BCNU (10 mg/kg) is presumed to be a result of the toxicity associated with the 10 mg/kg dose of BCNU in SCID mice as there was in excess of 15% body weight loss in the BCNU alone or BCNU plus O4BF groups. This can alter drug distribution due to the effect of dehydration which often accompanies the weight loss occurring with cytotoxic drug therapy.

9. I hereby declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

4/9/09
Date

Melinda Hollingshead, D.V.M., Ph.D.

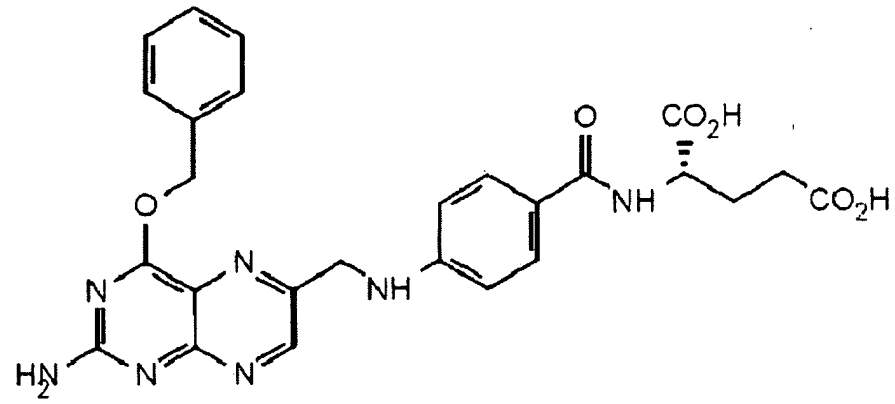

Figure 1: NSC 730793 O^4-benzylfolic acid (O4BFA)

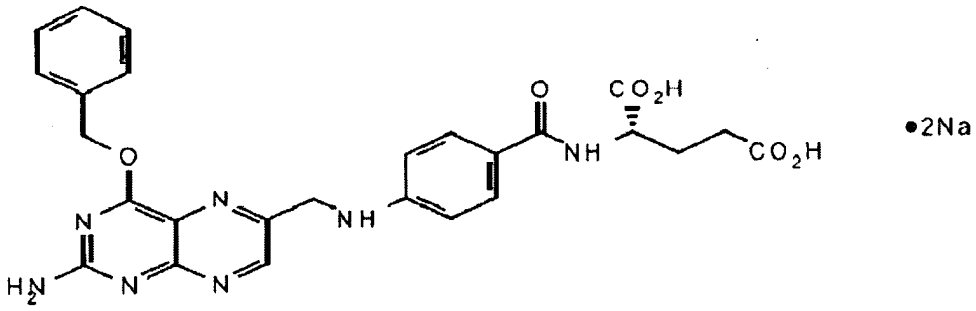

Figure 2: NSC 742482 O^4-benzylfolate disodium salt trihydrate (O4BF)

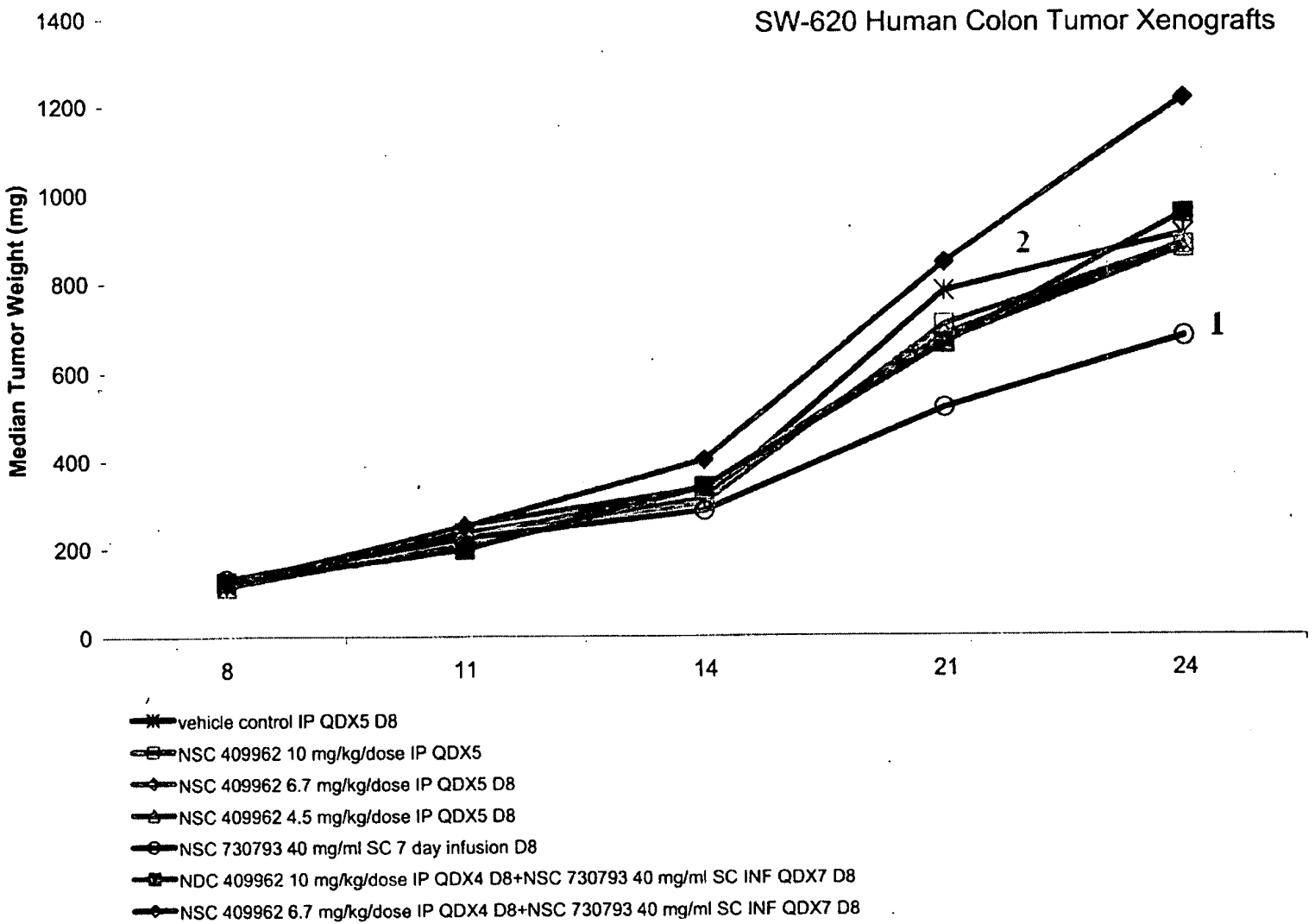
SW-620 Human Colon Tumor Xenografts
Median Tumor Weight (mg)
1400
1200
1000
800
600
400
200
0
8
11
14
21
24
2
1
vehicle control IP QDX5 D8
NSC 409962 10 mg/kg/dose IP QDX5
NSC 409962 6.7 mg/kg/dose IP QDX5 D8
NSC 409962 4.5 mg/kg/dose IP QDX5 D8
NSC 730793 40 mg/ml SC 7 day infusion D8
NDC 409962 10 mg/kg/dose IP QDX4 D8+NSC 730793 40 mg/ml SC INF QDX7 D8
NSC 409962 6.7 mg/kg/dose IP QDX4 D8+NSC 730793 40 mg/ml SC INF QDX7 D8

Figure 3

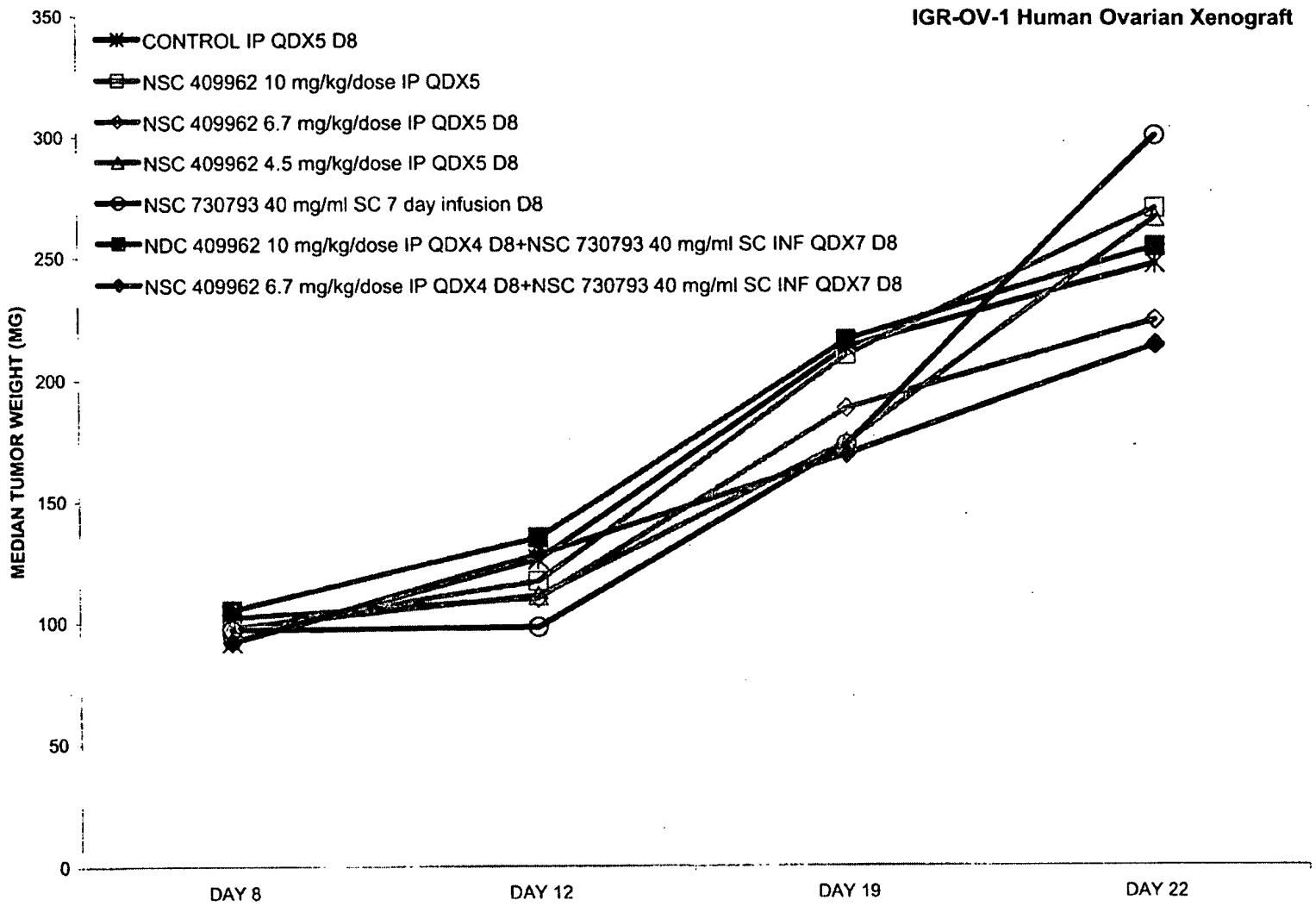
IGR-OV-1 Human Ovarian Xenograft
CONTROL IP QDX5 D8
NSC 409962 10 mg/kg/dose IP QDX5
NSC 409962 6.7 mg/kg/dose IP QDX5 D8
NSC 409962 4.5 mg/kg/dose IP QDX5 D8
NSC 730793 40 mg/ml SC 7 day infusion D8
NDC 409962 10 mg/kg/dose IP QDX4 D8+NSC 730793 40 mg/ml SC INF QDX7 D8
NSC 409962 6.7 mg/kg/dose IP QDX4 D8+NSC 730793 40 mg/ml SC INF QDX7 D8
MEDIAN TUMOR WEIGHT (MG)
350
300
250
200
150
100
50
0
DAY 8
DAY 12
DAY 19
DAY 22

Figure 4

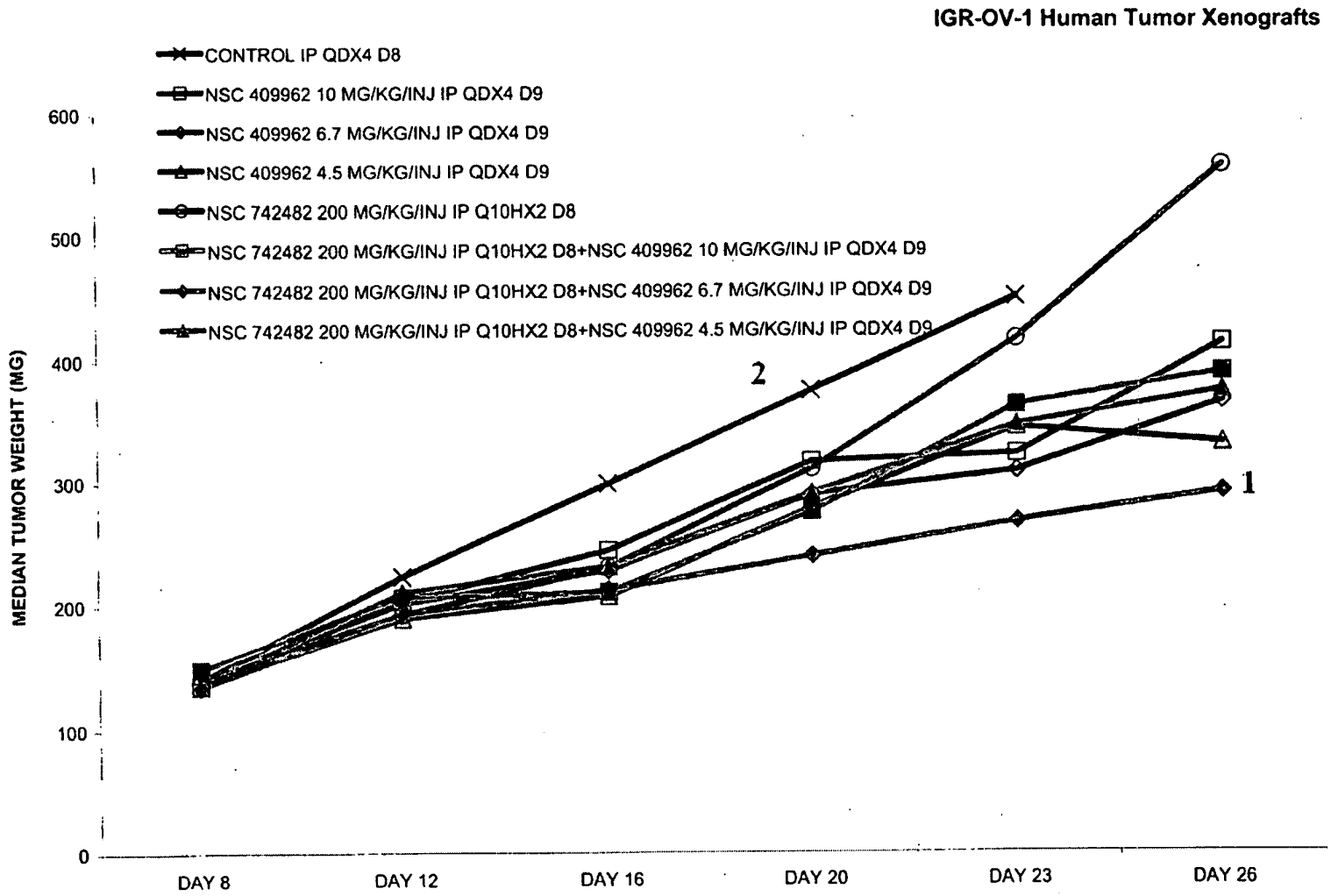
IGR-OV-1 Human Tumor Xenografts
CONTROL IP QDX4 D8
NSC 409962 10 MG/KG/INJ IP QDX4 D9
NSC 409962 6.7 MG/KG/INJ IP QDX4 D9
NSC 409962 4.5 MG/KG/INJ IP QDX4 D9
NSC 742482 200 MG/KG/INJ IP Q10HX2 D8
NSC 742482 200 MG/KG/INJ IP Q10HX2 D8+NSC 409962 10 MG/KG/INJ IP QDX4 D9
NSC 742482 200 MG/KG/INJ IP Q10HX2 D8+NSC 409962 6.7 MG/KG/INJ IP QDX4 D9
NSC 742482 200 MG/KG/INJ IP Q10HX2 D8+NSC 409962 4.5 MG/KG/INJ IP QDX4 D9
MEDIAN TUMOR WEIGHT (MG)
600
500
400
300
200
100
0
DAY 8
DAY 12
DAY 16
DAY 20
DAY 23
DAY 26
2
1

Figure 5